PRESERVATION TECHNIQUES FOR SCARABAEID AND OTHER INSECT LARVAE.

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Synopsis.

Methods commonly employed in preserving insect larvae are compared and the use, especially for scarabaeid larvae, of Peterson's "KAAD" fixative is recommended. The various fixatives in use are compared in regard to price; a modified cheaper form of Peterson's fixative, giving equally good results, is described.

The probable function of the fixative components is discussed and evidence recorded which suggests that the discoloration of preserved larvae is due to tyrosinase activity; *in vivo,* the tyrosinase is inhibited by a dehydrogenase system. The very rapid distension of larvae killed in KAAD or its modification is discussed in the light of recent work on insect cuticle.

Detailed recommendations are given for the cleaning, fixing and storage of larval Scarabaeidae.

Introduction.

The writer has experimented with a variety of methods of preserving insect larvae. Much of his earlier collected material, and of that obtained from the collections of earlier workers, shows marked faults, resulting from the use of unsatisfactory methods of preservation. While ethyl alcohol is used almost universally as a storage fluid, previous fixation is essential with most species; larvae killed and stored directly in alcohol become blackened and distorted and almost valueless for scientific study.

The purpose of this paper is to compare preservation techniques in common use, to bring to notice a valuable new fixative devised by Alvah Peterson (Peterson, 1943, 1948), and to discuss the functions of the constituents of the latter.

Definition of a Satisfactory Fixative.

An ideal fixative would have the following characteristics:

(*a*) Larvae fixed in it should be distended and turgid, neither soft and flaccid, nor hardened and shrivelled. (*b*) The larvae so fixed should retain normal, or close to normal, coloration (Lepidopterous larvae present much greater difficulties in this regard than do Scarabaeid larvae), with no darkening of the softer parts, nor bleaching of the head capsule. An increase in body opacity is desirable for morphological work where setal patterns are to be studied. (*c*) The fixative fluid should be reasonably inexpensive, as large quantities are used in the field collecting of larvae; and (*d*) the fixative should be one which may be used cold, and in which the larvae may be held for prolonged periods without deterioration. This is particularly desirable on collecting expeditions, when regular transference of larvae from fixative to storage fluid is sometimes inconvenient.

Preservation Techniques Compared.

The bulk of the larval collections seen by the writer have been treated by one or other of the following methods. The following comments are based upon a critical comparison of these methods, using series of larvae of *Adoryphorus couloni* Blackb., *Semanopterus* sp. and *Sericesthis* sp., and on the writer's general observations on the preservation of a wide range of species of soil-inhabiting larvae. The compositions of the fixatives are given below.

a. By direct killing in a storage fluid of 70–95 per cent. ethyl alcohol or in methylated spirits.

This technique, which appears to be frequently used, is most unsatisfactory for scarab larvae. Darkening of the cuticle begins within 24 hours of killing, and older specimens are completely blackened, and may become either very soft or hardened, according to the strength of alcohol used.

b. By direct killing in a storage fluid of 4 per cent. formalin.

This technique is equally unsatisfactory, severe discoloration occurring in time. Formaldehyde vapour from the specimens is a continual source of irritation to the eyes during their examination.

c. By killing and fixing in boiling water, and subsequent storage in 70–95 per cent. ethyl alcohol, or in 4 per cent. formalin.

Quite good results can be obtained by this method, and darkening on storage is prevented to a great extent. Small larvae appear to respond better than large larvae, which may fail to become, or to remain, turgid. Collapse is frequent if the larvae are held in actively boiling water; much better results are obtained if the larvae are placed in a beaker of boiling water, which is then allowed to cool. If the high temperature is maintained, the fat lining the body wall is melted, and is deposited about the gut. Any heat treatment is undesirable when larvae are to be dissected.

d. Killing and fixing in one of the acetic-formalin fixatives, e.g., Carls, Blés, or Bouin, and transfer to 70 per cent. ethyl alcohol.

Carls' or Blés' fixatives can give good results in the laboratory where careful attention can be given to time and temperature of fixation. Overfixation, resulting in hardening and buckling of the body wall, is the common error. Such overfixation makes examination of setal patterns extremely difficult, and special "renovation" techniques may be necessary before determination of the larva is possible. These fixatives can be used cold for longer periods, but larvae should be sorted into size groups, because in the period required to ensure adequate fixation of large larvae, small larvae in the same series becomes grossly overfixed. However, where collections are being made at a number of localities, this introduces difficulties in that the number of containers to be carried is multiplied.

Bouin easily results in overfixation, and imparts an undesirable picric staining to the larvae, which is difficult to remove.

e. Killing and fixing in Carnoy's fixative and storage in 90 per cent. ethyl alcohol.

Killing and fixing in cold Carnoy gives excellent results, although larvae become somewhat soft and transparent if allowed to remain in the fixative for more than a few days.

Larvae fixed by methods *a–d* invariably die with their mandibles closely opposed or overlapping, and these cannot be moved apart, the articulations becoming rigid, so that dissection of the mouthparts is difficult. On the other hand larvae fixed in Carnoy die with the mandibles opposed, but the articulations remain quite flexible and are easily dissected.

f. Killing and fixing in Peterson's KAAD fixative, or derivatives thereof, and storing in 95 per cent. ethyl alcohol.

The use of Peterson's KAAD results in larvae in an ideal state of preservation. The larvae are well distended, firm and completely free of any discoloration. There is an increase in opacity of the body wall.

Although Peterson states that best results are obtained by fixing for periods not longer than four hours, the writer has not observed any deterioration when larvae are left in the fixative for periods of up to three weeks. Best results for scarab larvae are given by fixation for not less than 2–3 hours.

As the writer's use of KAAD began only two months prior to the drafting of this paper, he has not seen larvae so preserved for periods longer than this. It is the writer's observation that, with all other preservation techniques, any tendency towards deterioration becomes evident within a week of treatment. Peterson states that the larvae retain their good condition for "a prolonged period".

Larvae killed in KAAD almost invariably die with the mandibles apart, the articulations remaining flexible. Important taxonomic characters occur on the mandibles, and dissection may often be avoided when the mandibles remain apart.

The fixative is expensive, containing approximately 8 per cent. dioxane. For this reason the writer has tried omitting this component, with results equal to those

obtained with the complete fixative. The acetic acid content may be reduced to 25 per cent. of that recommended by Peterson, or may be omitted altogether if the larvae are previously killed in hot water.

For purposes of gross dissection, larvae fixed in KAAD are considerably superior to those prepared by any of the other methods described. Dr. M. F. Day, of the Division of Entomology, C.S.I.R.O., has kindly examined mid-gut tissues of cetoniid larvae so treated. He found that histologically the mid-gut was fairly well preserved, considering the gross method of fixation employed, and that staining of the tissues was satisfactory.

Discussion.

The writer has rarely seen any discussion of the possible function of components of fixative mixtures, and has attempted to gain some understanding of these functions.

Firstly, all fixatives known to the writer contain acetic acid and alcohol. It is generally stated that acetic acid, together with the alcohol, is responsible for precipitation ("fixation") of the body protein, and that for most larvae its presence is necessary to prevent subsequent blackening.

The fact that hot water treatment prevents discoloration suggests that the blackening is an enzymatic process. That the enzyme is probably tyrosinase is supported by the following observations. Blackening is prevented by acids, suggesting that inactivation of the enzyme results from denaturation of the protein portion of the enzyme. Tyrosinase is known to contain copper in its prosthetic group and to be inactivated by cyanide. Larvae killed in cyanide do not blacken when stored in alcohol but will do so if first placed in an alcoholic solution of cupric chloride. The period of immersion in hot water necessary to destroy the enzyme varies from one minute at 100°C. to 30 minutes at 55°C. with *Semanopterus* sp.

The rapidity with which larvae blacken depends upon the treatment, which suggests that there is some system present which prevents blackening. Dennell (1949) considers that the tyrosinase in the larvae of *Calliphora erythrocephala* is prevented from acting upon its substrate by the presence of a dehydrogenase system which maintains a low redox potential in the insect tissues. Destruction of this system allows the redox potential to rise and the tyrosinase to act upon its substrate. Chloroform inhibits dehydrogenase systems and *Adoryphorus* and *Sericesthis* larvae placed in chloroform vapour are completely blackened an hour after anæsthesia. When killed in ethyl acetate vapour these larvae show no trace of blackening in the same period of time, which suggests the presence in these larvae of a tyrosinase-inhibiting dehydrogenase system.

Hurst (1940) has observed that a polar substance of low dielectric constant such as alcohol, is greatly assisted in its passage through the lipoid layer of insect cuticle by the presence of a non-polar compound of high dielectric constant, such as kerosene. The very rapid distension of larvae killed in Peterson's "KAAD" appears to be due to this phenomenon. Scarab larvae placed in either ethyl alcohol or kerosene die very slowly, and distension takes place very slowly. Death occurs very rapidly in a mixture of these two substances, and larvae are fully distended in less than an hour. The rate of entry of alcohol may be reduced by lowering the proportion of kerosene in the mixture, and Peterson found this necessary with some soft-bodied larvae, which otherwise burst before equilibrium was established.

Some kerosenes are not completely miscible with alcohol-acetic acid mixtures, and Peterson finds that adding dioxane results in complete miscibility. While Peterson considers that the dioxane itself may improve the quality of some larvae, the writer has found larvae preserved in KAA equally good. The KAA becomes cloudy during fixation to a much greater extent than does KAAD, although the cloudiness may be greatly reduced by the use of absolute rather than 95 per cent. alcohol in preparing the fixative. The fixative may be filtered and used again a number of times.

Composition and Estimated Costs of Fixatives.*

Bouin.—Picric sat. aqueous soln. 71 per cent., formalin 24 per cent., glacial acetic 3 per cent., approx. 7s. per gallon.

Carls.—Water 57 per cent., absolute alcohol 28 per cent., formalin 11 per cent., glacial acetic 4 per cent., approx. 4s. per gallon.

Blés.—70 per cent. alcohol 90 per cent., formalin 7 per cent., glacial acetic 3 per cent., approx. 3s. 6d. per gallon.

Carnoy.—Absolute alcohol 60 per cent., chloroform 30 per cent., glacial acetic 10 per cent., approx. £1 1s. per gallon.

KAAD.—Kerosene 8 per cent., 95 per cent. alcohol 70 per cent., glacial acetic 14 per cent., dioxane 8 per cent., approx. £1 per gallon.

KAA(1).—Kerosene 8 per cent., 95 per cent. alcohol 77 per cent., glacial acetic acid 15 per cent., approx. 9s. per gallon.

KAA(2) (with acetic acid content reduced).—Kerosene 8 per cent., 95 per cent. alcohol 87 per cent., glacial acetic 5 per cent., approx. 5s. per gallon.

While there is probably little to choose between Carnoy and KAA(2), it will be seen that the price of the former is approximately four times that of the latter.

Other Faults in Preserved Larvae.

When larvae are immersed in any fluid other than actively boiling water, regurgitation of part of the gut contents occurs. The black fluid coagulates on the mouthparts. The latter possess taxonomically important structures, which must be examined in detail for specific determination; the coagulated material must therefore first be removed. This is a tedious operation, and one likely to damage delicate structures: it may be made unnecessary either by starving the larvae for several days before killing, so that the regurgitated fluid is colourless and leaves no deposit, or the larvae may be anæsthetized before placing in the fixative, when no regurgitation occurs. Many scarab larvae are remarkably slow to succumb to cyanide, and the most rapid anæsthesia is brought about by carbon dioxide or chloroform. Deep anæsthesia is necessary, or the larvae recover sufficiently in cold fixative to regurgitate.

Tubes containing preserved larvae must be sealed to prevent evaporation, otherwise the percentage alcohol content of the fluid decreases rapidly, due to the higher volatility of alcohol over water in the mixture. Browning and changes of texture of the larvae then occur which are highly undesirable. If some evaporation does occur, the tube should be topped up with an alcohol of higher strength than that originally used.

Recommended Procedure for Preservation of Scarabaeid Larvae.†

(*a*) Handle larvae with blunt forceps, and remove superficial dirt by blowing, or by gentle washing in cold water. With some cetoniid larvae, a soft brush may be necessary to dislodge adhering soil. Such cleaning movements should be made in a caudal direction to avoid damage to the setae clothing the body.

(*b*) Anaesthetize the larvae deeply in carbon dioxide, or chloroform or ethyl acetate vapours.

(*c*) Kill and fix in Peterson's KAAD or KAA for not less than two hours.

(*d*) Wash larvae in alcohol briefly and store in 95 per cent. alcohol. Not more than two-thirds of the effective length of the tube should contain larvae, which should always be well covered by alcohol.

Larvae other than Scarabaeids.

Peterson's KAAD or KAA has been tried with a number of larval types. Excellent fixation was given with larvae of Carabidae, Elateridae, Cerambycidae, Chrysomelidae, Asilidae, and Lepidoptera. The fixative appears to be unsatisfactory for some Calliphorid larvae.

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* Based on Australian prices as at July, 1950.

† This refers to larvae killed in the laboratory. In the field, anæsthesia may have to be omitted, and cleaning postponed until transfer to storage fluid in the laboratory.

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References.

DENNELL, R., 1949.—Weismann's ring and the control of tyrosinase activity in the larva of *Calliphora erythrocephala*. *Proc. Roy. Soc. L.*, (B), 136 : 94.

HURST, H., 1940.—Permeability of insect cuticle. *Nature*, 145 : 462.

PETERSON, ALVAH, 1943.—Some new killing fluids for larvae of insects. *J. Econ. Ent.*, 36 : 115.

————, 1948.—Larvae of insects, Lepidoptera and Hymenoptera. Pt. 1. *Columbus, Ohio.*